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Antigenic Differences among *Campylobacter fetus* S-Layer Proteins

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of S-layer proteins extracted from *Campylobacter fetus* strains by using acid glycine buffer showed that the predominant S-layer proteins of different strains had subunit molecular weights in the range of 90,000 to 140,000. Electron microscopy revealed oblique S-layer lattices with a spacing of approximately 5.6 nm (γ = 75°) on wild-type strains VC1, VC119, VC202, and VC203. Three variants of *C. fetus* VC119 producing a predominant S-layer subunit protein of different molecular weight (M_r) from that of the parent were also examined. Each variant produced an oblique lattice morphologically indistinguishable from that of the parent. Amino-terminal sequence analysis showed that the S-layer proteins of the VC119 parent and variants were identical up to residue 18 and that this sequence differed from but was related to the first 16 N-terminal residues shared by the S-layer proteins of the three other wild-type *C. fetus* isolates. Western immunoblot analysis with an antiserum prepared to the VC119 protein and an antiserum prepared to *C. fetus* 84-40 LP (Z. Pei, R. T. Ellison, R. V. Lewis, and M. J. Blaser, J. Biol. Chem. 263:6416–6420, 1988) showed that strains of *C. fetus* were capable of producing S-layer proteins with at least four different antigenic specificities. Immunoelectron microscopy with antiserum to the VC119 S-layer protein showed that *C. fetus* cultures contained cells with immunoreactive oblique S-layer lattices as well as cells with oblique S-layer lattices which did not bind antibody. This suggests that *C. fetus* S-layer proteins undergo antigenic variation. Thermal denaturation experiments indicated that the antigenicity conferred by the surface-exposed *C. fetus* S-layer epitopes was unusually resistant to heat, and the thermal stability appeared to be due to the highly organized lattice structure of the S layer. Protease digestion of purified VC119 S-layer protein revealed a trypsin-, chymotrypsin-, and endoproteinase Glu-C-resistant domain with an apparent M_r of 110,000, which carried the majority of the epitopes of the S-layer protein, and a small enzyme-sensitive domain. The trypsin- and chymotrypsin-resistant polypeptides shared an overlapping sequence which differed from the N-terminal sequence of the intact S-layer protein.

Campylobacter fetus is an economically significant veterinary pathogen. Two subspecies are classified, *C. fetus* subsp. *fetus* and *venerealis*. The two subspecies are indistinguishable on the basis of DNA-DNA reassociation and partial 16S rRNA sequence analysis, and the division into two subspecies is based on the ability to grow in the presence of 1% glycine and differences in pathogenesis (2, 39, 40, 46). *C. fetus* subsp. *fetus* causes sporadic abortion in cattle and sheep (30, 43), while *C. fetus* subsp. *venerealis* is the major cause of bovine genital campylobacteriosis, a venereally transmitted disease which can lead to infertility (46). *C. fetus* subsp. *fetus* infections of humans are rare, but because the organism becomes systemic, especially in immunocompromised individuals, *C. fetus* infections are generally serious in consequence (17, 19, 34, 37). Septicemia and meningitis are the most frequently diagnosed *C. fetus* infections, but pericarditis, peritonitis, salpingitis, septic arthritis, septic abortions, and abscesses have also been reported (17, 19, 34, 37). The ability of *C. fetus* to produce these diseases appears to be associated with the presence of a crystalline surface protein array, or S layer (7, 14, 29, 30, 32). S layers are regular, two-dimensional assemblies of protein monomers that constitute the outermost layer of the cell envelope of many bacteria (22, 45, 47). In the case of *C. fetus*, this structure makes the cell resistant to phagocytic uptake (8, 29) and to the bactericidal activity of serum (7, 8).

The *C. fetus* S layer has a fine delicate crystalline structure (14) and is composed of high-molecular-weight (M_r) protein subunits for which a range of M_rs between 98,000 and 149,000 has been reported (14, 30, 32, 33). Indeed, while Myers (32), McCoy et al. (30), and Dubreuil et al. (14) isolated only a single S-layer protein from the strains they examined, Pei et al. (33) isolated putative S-layer proteins with M_rs of 100,000 and 127,000 from a single strain of *C. fetus*. The N-terminal sequences of the S-layer proteins studied by Pei et al. (33) were identical with each other but differed from that of the *C. fetus* S-layer protein characterized by Dubreuil et al. (14). Interestingly, Pei et al. (33) also reported the presence of shared antigenic determinants in the proteins they examined and suggested that *C. fetus* produces a family of S-layer proteins with common structural and antigenic characteristics. Blaser et al. (7) have in fact suggested the S layer as a candidate for a *C. fetus* vaccine.

Other studies have indicated, however, that *C. fetus* can undergo antigenic changes during the course of an infection. For example, Schurig et al. (42, 44) reported that the emergence of antigenic variants is a general consequence of *C. fetus* infection in cattle, while Corbeil et al. (10) suggested that changes in antigenic structure provide a mechanism which facilitates the maintenance of an asymptomatic cervicovaginal carrier state in cattle. Schurig et al. (43) also demonstrated in vivo changes in serotype during persistent infections with an intestinal strain of *C. fetus*. The O antigen of *C. fetus* does not undergo antigenic variation (10, 42), and the antigenic variation seen during the course of *C. fetus* infections involves superficial protein antigens. The immu-

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nodominant protein antigen of the *C. fetus* cell is the so-called microcapsule or S layer (14, 33), and Schurig et al. (42) suggested that strains of *C. fetus* may have the capacity to produce microcapsules of varied antigenic structure and that during infection selection occurs for variants expressing a microcapsule differing antigenically from that of the infecting parent strain.

To investigate the ability of *C. fetus* to produce antigenically different S-layer proteins, we identified strains of *C. fetus* which produced S-layer proteins of differing subunit M_r and had the two different reported N-terminal amino acid sequences. We also isolated clonal variants of one strain which produced S-layer proteins with subunit M_rs different from that of the parent. Using Western immunoblotting with antisera prepared against two different *C. fetus* S-layer proteins, we examined the antigenic differences of *C. fetus* S-layer proteins. We also examined the morphology and immunoreactivity of the native *C. fetus* S layers. In addition, we demonstrated the thermal stability of *C. fetus* S-layer antigenicity and used protease cleavage to identify the epitope-bearing domain of the *C. fetus* S-layer protein. Here we report our findings.

MATERIALS AND METHODS

Bacterial strains and growth conditions. S-layer-producing *C. fetus* subsp. *fetus* strains VC1, VC119, VC202, and VC203 were supplied by P. L. Stovell, Animal Pathology Laboratory, Vancouver, British Columbia, Canada. Three spontaneous S-layer variants of strain VC119 (VC119-A2, VC119-A3, and VC119-B2) isolated in our laboratory were also examined. These variants produced smaller colonies than VC119, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell lysates showed that they produced an S-layer protein with a different apparent M_r from that of VC119. The S-layer-negative strain VC78 (NCTC 10842; National Collection of Type Cultures, Collindale, United Kingdom) was also used. The subspecies of all strains and variants was confirmed by their ability to grow in the presence of 1% glycine. Stock cultures were maintained at −70°C in tryptic soy broth (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 15% (vol/vol) glycerol. Cultures were grown for 48 h in an anaerobic jar at 37°C on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.). An atmosphere containing 5% oxygen and 10% CO_2 was produced with a gas generation kit for campylobacters (Oxoid Ltd., Basingstoke, England).

Water and glycine extraction of S-layer proteins. Extraction of S-layer proteins from cells grown for 48 h was done by the method of Pei et al. (33) and by the method of McCoy et al. (30), modified as previously described (14).

Membrane and S-layer preparations. For electron microscopic examination of S-layer morphology, cells were harvested from plates into 20 mM Tris hydrochloride (pH 7.5), collected by centrifugation at 4,000 × *g* for 30 min, suspended in 25% (wt/vol) sucrose in 50 mM Tris hydrochloride (pH 8.0), and treated on ice with 0.5 volume of 10 mg of lysozyme per ml in 50 mM Tris hydrochloride (pH 8.0). The cells were then lysed by the addition of 0.1 volume of 10% (vol/vol) sarcosyl in 50 mM Tris hydrochloride (pH 8.0). DNase and RNase were added to final concentrations of 20 μg/ml each, and outer membranes were collected by centrifugation at 13,000 × *g* for 15 min.

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (23) with a mini-slab apparatus (Bio-Rad Laboratories, Richmond, Calif.). Protein solubilized in sample buffer was stacked in a 4.5% acrylamide gel (100-V constant voltage) and separated with a 7.5% acrylamide gel (200 V). Protein was stained with Coomassie blue R-250.

When required, separated proteins were transferred from the slab to nitrocellulose paper (NCP) by the methanol Tris-glycine method described by Towbin et al. (49). Electroblotting was carried out in a Trans-blot apparatus (Bio-Rad) for 18 h at 60 V.

N-terminal sequence analysis. The S-layer protein of *C. fetus* VC119 was purified by fast protein liquid chromatography as previously described (14). The predominant S-layer proteins in glycine extracts of other strains of *C. fetus* and variants of *C. fetus* VC119 were purified by migrating a glycine extract of the cells on a 7.5% acrylamide gel and electroblotting to Immobilon transfer membranes (Millipore Corp., Bedford, Mass.) by the method of LeGendre and Matsudaira (26). The blotted proteins were stained with Ponceau S (Sigma Chemical Co., St. Louis, Mo.) by the method of Salinovich and Montelaro (41). Amino acid sequencing was performed on a model 475A protein sequencing system (Applied Biosystems, Inc., Foster City, Calif.) running a standard operating program. Phenylthiohydantoin derivatives were separated with an on-line phenylthiohydantoin analyzer (model 120A; Applied Biosystems). Data handling was done with a data analyzer (model 900 control/data analysis module; Applied Biosystems).

Protease treatments. Purified *C. fetus* VC119 S-layer protein was prepared at a concentration of 1 mg/ml in 20 mM Tris hydrochloride (pH 8.0). *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated alpha-chymotrypsin (Sigma), or endoproteinase Glu-C (*Staphylococcus aureus* V8 protease; Boehringer GmbH, Mannheim, Federal Republic of Germany) was added at 200, 10, or 100 μg/ml, respectively; samples were incubated at 37°C for 60 min and analyzed by SDS-PAGE. In the case of the trypsin digestion, soybean trypsin inhibitor (Sigma) was added at 400 μg/ml to terminate the reaction.

Purification of protease digestion fragments. Protease digests of purified *C. fetus* VC119 S-layer protein were loaded onto a Superose 12 column (Pharmacia, Uppsala, Sweden). The fragments were purified by fast protein liquid chromatography with 20 mM Tris hydrochloride (pH 7.4) containing 0.1 M NaCl at a flow rate of 0.5 ml/min. The eluant was monitored at 230 nm, and the fractions were analyzed by SDS-PAGE.

Antibody production. Antiserum VC119-DD4 was raised in an adult New Zealand White rabbit by intramuscular injection with 50 μg of fast protein liquid chromatography-purified *C. fetus* VC119 S-layer protein in Freund complete adjuvant. Booster doses were given in Freund incomplete adjuvant on days 14 and 28. On day 42, the rabbit was exsanguinated and the serum was collected and stored at −20°C. Control nonimmune serum was obtained before the first injection. Antibodies to lipopolysaccharide were removed by absorption with S-layer-negative strain VC78. This strain produced lipopolysaccharide of the same serogroup as strain VC119. Antiserum 84-40 LP-MB1 to the M_r 100,000 protein of strain 82-40 LP was graciously supplied by M. Blaser.

Absorption of antisera. Cells from a 24-h culture (approximately 10 mg, either boiled or unboiled) were mixed with an equal volume of serum and incubated at room temperature for 1 h. Antibodies bound to surface epitopes were removed by centrifugation at 15,000 × *g* for 10 min, and the serum was reabsorbed with the appropriate antigen suspension.

The procedure was repeated, and absorbed antisera were stored at −20°C. In certain cases, cells grown for 48 h were suspended in phosphate-buffered saline (pH 7.4) and boiled for 1 h in the absence or presence of 0.1 or 0.5% (wt/vol) SDS (Serva, Heidelberg, Federal Republic of Germany). After this treatment, cells were collected by centrifugation at 15,000 × *g* for 10 min.

Western blotting. After electroblotting, unreacted sites on the NCP were blocked with a 1% (wt/vol) solution of gelatin in 10 mM Tris hydrochloride–0.9% NaCl (pH 7.4) (GTS buffer) for 1 h at room temperature. The NCP was then incubated with an appropriate dilution of antiserum in the same buffer for 2 h. The NCP was washed five times with Tris-saline (10 mM Tris hydrochloride, 0.9% NaCl [pH 7.4]). Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (CALTAG Laboratories, South San Francisco, Calif.) was then added in GTS buffer and incubated for 1 h at room temperature. After incubation, the NCP was washed five times in Tris-saline. The reactive bands were visualized as described by Blake et al. (6), with 5-bromo-4-chloro-3-indolylphosphate (Boehringer) as the alkaline phosphatase substrate and Nitro Blue Tetrazolium (Sigma) as the color development reagent.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was done essentially as described by Engvall and Perlmann (15). Antigen was tested in triplicate at 5 μg per well, and the developing antibody was goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (CALTAG). The A_{405} was read with an enzyme immunoassay model 310 ELISA reader (Bio-Tek Instruments, Inc., Highland Park, Vt.). Bovine serum albumin (BSA) was used as the antigen negative control.

Electron microscopy. A grid covered with a Formvar film was floated on a 50-μl drop of bacterial cells suspended in 100 mM Tris (pH 7.5) containing 150 mM NaCl, 0.05% Tween 20, and 0.5% BSA (Tris-NaCl-TW-BSA) for 5 min. For negative staining, the grids were stained by floating on a drop of 1% (wt/vol) ammonium molybdate containing 0.1% (vol/vol) glycerol (pH 7.5) and were examined in an EM-300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.) operated at an accelerating voltage of 60 kV. Images were recorded on 70-mm Fine Grain Release Film (Eastman Kodak Co., Rochester, N.Y.). For immunogold electron microscopy, the grid was then removed from the drop of bacterial cells and floated on a drop of Tris-NaCl-TW-BSA containing 10% (vol/vol) antiserum. After 2 h of incubation, the grid was removed, and nonspecifically bound immunoglobulin G was removed by floating the grid on two drops of Tris-NaCl-TW-BSA. The grid was then floated on a drop of Tris-NaCl-TW-BSA containing a 1:10 dilution of 15-nm colloidal gold particles coated with protein A (Janssen Biotech NV, Olen, Belgium). After incubation for 1 h, the nonspecifically bound colloidal gold particles were removed by floating the grid on two drops of Tris-NaCl-TW-BSA and one drop of distilled water. The grids were negatively stained and examined as described above.

RESULTS

Morphological and biochemical analysis. When the distilled water extraction procedure described by Pei et al. (33) or the acid glycine buffer extraction procedure originally described by McCoy et al. (30) was applied to S-layer-producing strains of *C. fetus* subsp. *fetus* from a variety of sources, SDS-PAGE analysis of the glycine extracts showed that the M_r of the predominant S-layer subunit protein differed

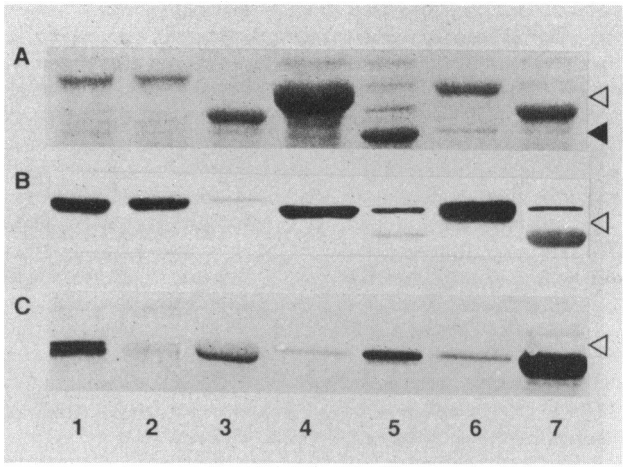

FIG. 1. (A) SDS-PAGE (7.5% polyacrylamide) of glycine-extracted S-layer proteins of *C. fetus* strained with Coomassie blue. (B) Western blot of SDS-PAGE (12.5% polyacrylamide)-separated glycine-extracted S-layer proteins of *C. fetus* reacted with a 1:1,000 dilution of antiserum raised against purified *C. fetus* VC119 S-layer protein. (C) Western blot of SDS-PAGE (12.5% polyacrylamide)-separated glycine-extracted S-layer proteins of *C. fetus* reacted with a 1:1,000 dilution of antiserum 84-40 LP-MB1 raised against purified *C. fetus* 84-40 LP protein. Lanes in all panels: 1, VC1; 2, VC202; 3, VC203; 4, VC119; 5, VC119A2; 6, VC119-B2; 7, VC119-A3. The open arrowheads indicate an M_r of 116,250, and the closed arrowhead in panel A indicates an M_r of 97,400.

depending on the strain being examined (Fig. 1A). In the case of *C. fetus* VC1 and VC202, this predominant S-layer protein displayed an M_r of approximately 140,000, while the reference strain of our laboratory, VC119, had an S-layer subunit with an apparent M_r of 135,000. In the case of *C. fetus* VC203, the predominant S-layer protein had an apparent M_r of 110,000, although minor higher-M_r proteins were also present. Electron microscopy showed an oblique S layer (Fig. 2A) with a lattice spacing of approximately 5.6 nm and an angle of 75° between lattice vectors (Fig. 2B). The best images of the *C. fetus* S layer were obtained by negative staining with 1% (wt/vol) ammonium molybdate containing 0.1% (vol/vol) glycerol, and the best order was seen on preparations of sarcosyl-extracted cell envelopes (Fig. 2B). However, unlike many other S layers, good order was shown only in patches. This was especially true when the layer was examined on the cell surface or on membrane blebs (Fig. 2C). Sheets of S layer were seldom observed, and when seen on the surface of an outer membrane bleb, the S-layer subunits followed the curvature of the bleb, indicating that the subunits were loosely held together (Fig. 2B, inset). The side view of the subunits on the blebs showed that the height of the layer subunits (approximately 11 nm) was greater than the center-to-center spacing of the array (5.6 nm). The estimate of the height of the subunits should be considered an upper limit of the possible height, as the superimposition of rows of proteins at different tilt angles may give an overestimate of the actual height of the protein.

Three variants of *C. fetus* VC119 that produced S-layer protein subunits which had M_rs different from the M_r of 135,000 of the S-layer subunit of parent strain VC119 were also examined. SDS-PAGE analysis showed that the predominant glycine-extractable protein of variant VC119-A2 had the lowest apparent M_r of the *C. fetus* S-layer proteins examined in this study, with an apparent subunit M_r of 85,000 (Fig. 1A, lane 5). The apparent M_r of the predominant

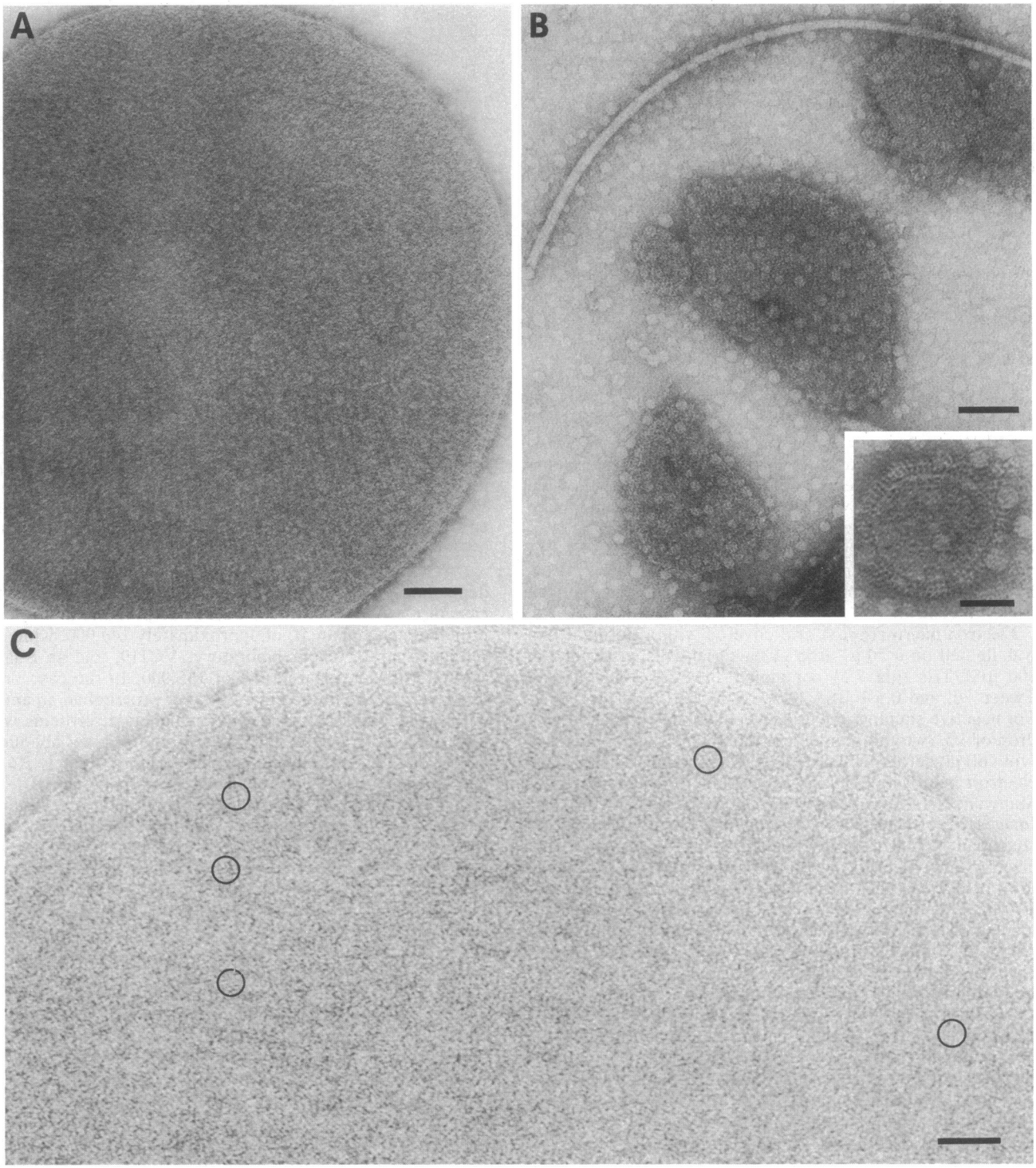

FIG. 2. Electron micrographs of *C. fetus* S layers negatively stained with 1% (wt/vol) ammonium molybdate. (A) Membrane bleb from *C. fetus* VC1 covered with S-layer protein. Bar = 100 nm. (B) Sarcosyl-extracted cell envelopes of *C. fetus* VC119 showing S layer arranged as an oblique lattice. Bar = 100 nm. The inset shows a profile of the S-layer morphological units. The repeat spacing is 5.6 nm, and the height of the units is approximately 11 nm on an outer membrane bleb. Bar in inset = 50 nm. (C) Higher magnification of area in panel A at higher contrast showing the general absence of good order in the *C. fetus* S layer when visualized on the surface of membrane blebs. Some areas showing a higher degree of order of the lattice are circled. Bar = 50 nm.

TABLE 1. N-terminal amino acid sequences of *C. fetus* S-layer proteins and peptides

Strain(s)	Amino acid residue[a]
	10 20
VC1, VC202, VC203	MLNKTDVSML YITIMGMASE
82-40, 84-112[b]	MLNKTDVSML YITIMGMASE
VC119,[c] VC119-A2, VC119-A3, VC119-B2	MISKSEVSEL FIVLFGRP
VC119 peptides	
Trypsin fragment	ESIDEAGLN KIALTTKNDT ITGE
Chymotrypsin fragment	KESIDEAGLN KIALTTKN

[a] Identical residues in intact protein sequences are underlined.
[b] Pei et al. (33).
[c] Dubreuil et al. (14).

glycine-extractable subunit of variant VC119-A3 was 110,000 (Fig. 1A, lane 7), while the apparent M_r of the predominant glycine-extractable protein subunit of variant VC119-B2 was the least altered at 130,000 to 133,000 (Fig. 1A, lane 6). In variants VC119-A2 and VC119-B2, minor high-M_r bands were also apparent at M_rs corresponding to the predominant protein of the various variants and the parent. For example, in the case of VC119-A2 (Fig. 1A, lane 5), the apparent M_rs of these minor bands were 110,000 and 130,000 to 133,000, with an additional band at 165,000. Variant VC119-A3 had a minor band at an apparent M_r of 165,000 (Fig. 1A, lane 7). In each variant, electron microscopy revealed the presence of an oblique S-layer lattice which was morphologically indistinguishable from that of the parent.

To evaluate the biochemical relatedness of the S-layer proteins which had different M_rs, small amounts of each predominant protein were purified by the Immobilon transfer method of LeGendre and Matsudaira (26), and N-terminal sequence analysis was performed. The N-terminal 20 amino acid residues of the proteins isolated from *C. fetus* VC1, VC202, and VC203 were identical to those reported by Pei et al. (33; N-terminal sequence 1) but differed from those of the proteins isolated from *C. fetus* VC119 and its variants (Table 1; N-terminal sequence 2). While the VC119 proteins had seven identical residues, including the N-terminal methionine, the VC119 proteins lacked the three additional methionine residues found in the S-layer proteins from the other *C. fetus* strains.

Immunochemical analysis. The antigenic cross-reactivity of the different *C. fetus* S-layer proteins was examined with two different antisera. Antiserum VC119-DD4 was prepared against the purified 135,000-M_r protein of *C. fetus* VC119 (N-terminal sequence 2). Antiserum 84-40 LP-MB1 was prepared to the 100,000-M_r putative S-layer protein of *C. fetus* 82-40, which shared N-terminal sequence 1 with the proteins of strains VC1, VC202, and VC203. The difference in antigenicity between the 135,000-M_r VC119 S-layer protein and the 100,000-M_r protein of strain 84-40 LP purified by Pei et al. (33) was shown when ELISAs were performed with antisera VC119-DD4 and 84-40 LP-MB1, with the purified VC119 135,000-M_r protein as a substrate. The results illustrated in Fig. 3A show that there was virtually no recognition of the VC119 protein by the 84-40 LP-MB1 antiserum.

Western blot analysis of glycine extracts with antiserum VC119-DD4 showed that the 140,000-M_r S-layer proteins of VC1 and VC202 shared cross-reactive epitopes with the

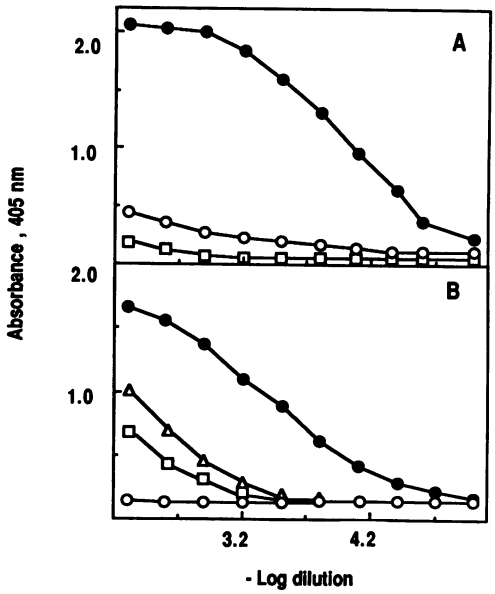

FIG. 3. ELISA of purified *C. fetus* VC119 S-layer protein (5 μg). (A) Reaction with homologous polyclonal antiserum VC119-DD4 against the purified VC119 protein, before (●) and after (□) boiling for 10 min. ○, Reaction of heterologous antiserum 84-40 LP-MB1 against the purified VC119 protein. (B) Reaction with polyclonal antiserum VC119-DD4 against the purified protein before (●) and after (○) absorption with boiled VC119 cells, after absorption with VC119 cells boiled in the presence of 0.1% SDS (□), and after absorption with VC119 cells boiled in the presence of 0.5% SDS (△).

VC119 protein (Fig. 1B). In the case of VC203, cross-reactivity was shown by a minor protein with an M_r of approximately 140,000, and no reaction was seen with the predominant 110,000-M_r protein. When the VC119 variants were examined, cross-reactivity was seen with a 135,000-M_r protein which was present as a minor component in VC119-A2 and VC119-A3 and as a predominant protein in VC119-B2. The predominant 110,000-M_r protein in strain VC119-A3 also showed antigenic cross-reactivity, but the predominant 85,000-M_r proteins of VC119-A2 showed little cross-reactivity with this antiserum. No reaction was seen with the minor band with an M_r of approximately 165,000 produced by the VC119 parent and variants (Fig. 1B).

When antiserum 84-40 LP-MB1 was used, a different antigenic reactivity profile was seen (Fig. 1C). No reaction was seen with the predominant 135,000- to 140,000-M_r proteins of strains VC1, VC202, VC119, VC119-A2, or VC119-B2. Strong antigenic cross-reactivity was, however, shown with minor proteins with M_rs of approximately 110,000 in strains VC1 and VC119-A2 and the predominant 110,000-M_r S-layer protein in VC119-B2. Reduced cross-reactivity was also shown with the predominant 110,000-M_r protein in strain VC203 and a minor 110,000-M_r protein in strain VC119-A3, and markedly reduced antigenic cross-reactivity was shown with a minor 110,000-M_r protein in strains VC202 and VC119.

Immunogold electron microscopy of strain VC119 with antiserum VC119-DD4 showed antibody binding to the surface of the oblique S-layer lattice (Fig. 4A). However, even in the case of the homologous strain, not all cells in the culture bound antibody. In the cases of variants VC119-A2, VC119-A3, and VC119-B2, many cells which possessed an oblique S-layer lattice were present in the culture, but the S

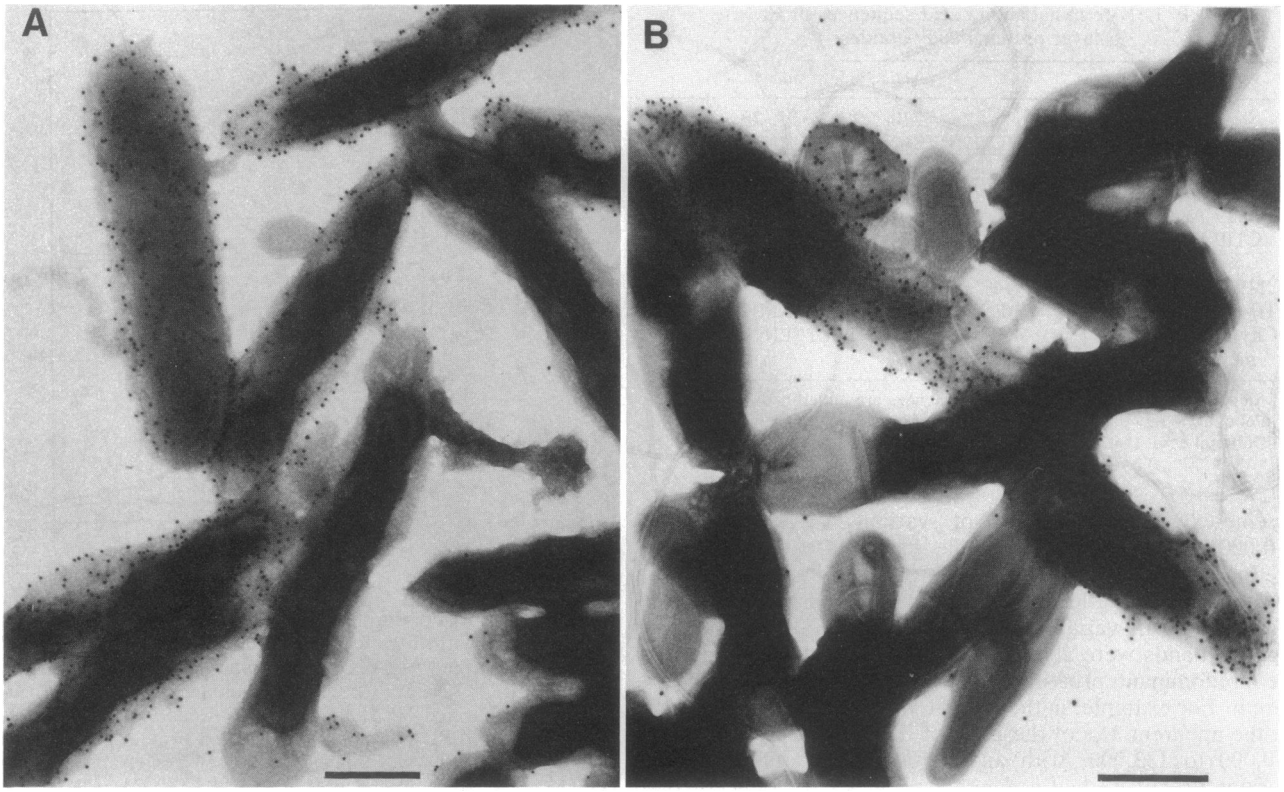

FIG. 4. Immunogold demonstration of intrastrain antigenic variation of the S layer, with a 1:10 dilution of polyclonal antiserum VC119-DD4 prepared against the purified VC119 S-layer protein. (A) *C. fetus* VC119, showing antibody labeling of many, but not all, cells. (B) *C. fetus* VC203, showing only a few cells labeled over the entire surface. Bar in each panel = 500 nm.

layer on these cells failed to bind antibody. Similarly, cultures of strains VC1, VC202, and VC203 (Fig. 4B) contained cells which bound antibodies to the VC119 S-layer protein and other cells which failed to bind antibodies. Under the conditions used, antibodies in antiserum 84-40 LP-MB1 appeared not to recognize surface epitopes on the S layer of any of the strains or variants examined.

The position of the immunodominant epitope-bearing domain of the *C. fetus* S-layer protein was investigated by protease digestion by using the VC119 protein as a model. When trypsin, alpha-chymotrypsin, or endoproteinase Glu-C was used to cleave the purified VC119 S-layer protein under nondenaturing conditions, a fragment with an M_r of approximately 110,000 was produced. This enzyme-resistant polypeptide was reactive by Western blotting (Fig. 5A), and in the case of the polypeptide produced by trypsin digestion, an ELISA showed that the enzyme-resistant polypeptide carried the majority of the epitopes of the intact VC119 S-layer protein (Fig. 5B). The immunodominant 110,000-M_r enzyme-resistant polypeptide of the VC119 S-layer protein was then purified by molecular sieving with a Superose 12 column and subjected to N-terminal amino acid sequence analysis. The trypsin- and chymotrypsin-generated peptides were overlapping except for the N-terminal lysine residue of the chymotrypsin-generated fragment. The overlapping sequence differed from the amino-terminal sequence of the intact protein, indicating that the two enzymes cleaved internally. The amino-terminal sequence of the endoproteinase Glu-C-generated fragment appeared to be blocked and could not be determined.

The thermal stability of the immunodominant epitopes of the *C. fetus* S layer was also investigated by using strain VC119 as a model. Absorption of antiserum to the purified VC119 S-layer protein with boiled VC119 cells removed essentially all antibody reactivity to the VC119 S-layer protein, as measured by ELISA (Fig. 3B), indicating that the immunodominant epitopes of the VC119 S-layer were unusually heat resistant. Immunogold electron microscopy confirmed the ability of the S-layer on boiled cells to bind antibody (data not shown). However, when the purified S-layer protein was boiled for 10 min and used as the antigen, an ELISA showed that the heated protein lost antigenic reactivity (Fig. 3A). This suggested that the heat stability of epitopes on the *C. fetus* S layer was a result in part of the organizational integrity of the assembled S-layer. To test this hypothesis, SDS was added to the cell suspension before boiling to facilitate disruption of S-layer organization. The results in Fig. 3B show that when cells of VC119 were boiled in the presence of SDS and used for absorption, there was an increase in the thermal sensitivity of the S-layer epitopes, as indicated by the smaller decrease in ELISA titer compared with that resulting from cells boiled in the absence of detergent. Also, an increase in SDS concentration from 0.1% to 0.5% (wt/vol) increased the thermal sensitivity of the VC119 S-layer epitopes, again consistent with increased disruption of S-layer integrity.

DISCUSSION

Given the surface location of S layers on bacterial cells, there have been surprisingly few studies on the antigenicity of their constituent proteins. In the case of pathogenic

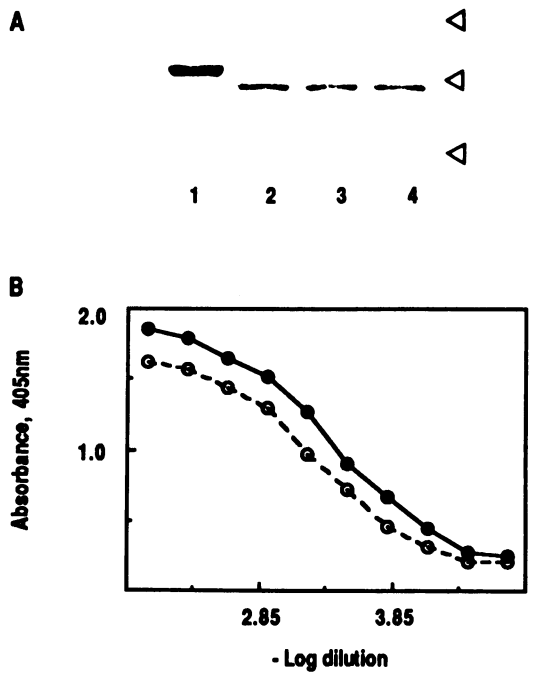

FIG. 5. (A) Western blot of purified S-layer protein of *C. fetus* VC119 (lane 1) and purified VC119 S-layer protein after digestion with trypsin (lane 2), alpha-chymotrypsin (lane 3), and endoproteinase Glu-C (lane 4) reacted with a 1:1,000 dilution of polyclonal antiserum VC119-DD4 prepared against the purified VC119 S-layer protein. The arrowheads indicate M_rs of 200,000, 116,250, and 66,200. (B) ELISA of 5 μg of purified *C. fetus* VC119 S-layer protein (●) and 5 μg of fast protein liquid chromatography-purified 110,000-M_r trypsin digest fragment of VC119 S-layer protein reacted with polyclonal antiserum VC119-DD4 against the purified VC119 protein (○).

bacteria, however, the S layer serves as the interface between the parasite and its host, and by virtue of its surface coverage, the S layer is generally the immunodominant protein antigen of the cell (14, 33). In the most-studied case, the so-called A layer of the fish pathogen *Aeromonas salmonicida*, Western blot analysis suggests that the antigenic structure of the S-layer protein is strongly conserved in strains isolated from diverse geographic sources and different fish species and causing different pathogeneses (36). The current study has shown that this is clearly not the situation in *C. fetus*. A single strain of *C. fetus* has the ability to both produce different antigenic types of S-layer subunit proteins and S-layer proteins with different subunit M_rs. In the case of *C. fetus* VC119, S-layer proteins with at least four different subunit M_rs (135,000, 130,000 to 133,000, 110,000, and 85,000) were produced, and three of these subunits were different antigenically. Indeed, among the strains and variants of *C. fetus* studied here, S-layer subunits of at least four different antigenicities were identified, one with cross-reactivity with the VC119 protein, one with cross-reactivity with the 84-40 LP protein, one with cross-reactivity with both of these proteins, and one with no apparent cross-reactivity with either protein. Immunogold electron microscopy further revealed that within a given culture of *C. fetus*, there were populations of cells with S layers which bound antibody to S-layer protein and cells with morphologically indistinguishable S-layers which failed to bind antibody. Taken together, these findings suggest that the S layer of *C. fetus* is subject to antigenic variation. This makes the *C. fetus* S layer unique among the S layers studied to date (45, 47) and likely explains the antigenic changes seen during the course of experimental and natural *C. fetus* infections of animals (10, 42–44).

In addition to this immunochemical information, the study has provided valuable insight into the structure of the *C. fetus* S-layer protein. On the basis of N-terminal sequence analysis, there appear to be at least two classes of *C. fetus* S-layer proteins, one corresponding to the sequence reported by Pei et al. (33) and represented here by the proteins isolated from strains VC1, VC202, and VC203 and the other corresponding to the sequence of the protein isolated from strain VC119 (14). However, although the two N-terminal sequences are different, they are clearly related. While the sequences have 7 identical residues in the first 18 residues, seven of the amino acid differences in the two sequences could result from single nucleotide changes. Indeed, by using the log-odds score of Dayhoff (11), the two different sequences displayed a mean homology score of 102. Virtually no identity is seen between either of the two *C. fetus* amino-terminal sequences and the sequences of other S-layer proteins that have been studied, however (16, 25, 35, 36, 50), and a search of the National Biomedical Research Foundation protein sequence library (27) failed to reveal any N-terminal sequences with significant homology to those of the *C. fetus* proteins, suggesting that they are proteins unique to this species.

All of the layers observed in this study displayed an oblique lattice, regardless of the N-terminal class of the protein or indeed the subunit M_r of the protein. Other investigators have reported observing hexagonal and tetragonal arrays on the surface of *C. fetus* (18, 29), and in the previous report on the S layer of strain VC119 (14), the morphology was not determined because the array could not be negatively stained. Whether the various reports by other workers reflect strain-to-strain differences in layer morphology in the case of *C. fetus* or are results of technical differences which provide for varying levels of resolution and hence diverse interpretations is not clear at this time. Certainly the *C. fetus* S layer has an extremely fine structure, and a high degree of order can be seen only in patches. Also, oblique arrays have not commonly been reported, and detailed descriptions have been provided only for two *Bacillus* species (31, 38) and *Aquaspirillum* (*Spirillum*) *putridiconchylium* (4, 5).

The ability of *C. fetus* VC119 and variant VC119-A2 to produce S-layer subunit proteins with subunit M_rs of 135,000 and 85,000, respectively, which assemble into morphologically indistinguishable arrays indicates at least in the case of the parent protein that there are regions of the *C. fetus* S-layer protein which are not essential for S-layer assembly and integrity. Indeed, in the case of these two organisms, a portion of polypeptide with an M_r of approximately 50,000 appears not to be essential for these functions. In the 135,000-M_r protein of VC119, this portion comprises the immunodominant region of the molecule, is surface exposed, and under nondenaturing conditions is protected from proteolytic cleavage. Sequence analysis further showed that changes giving rise to S-layer proteins with differences in subunit M_r and antigenicity occur downstream from the N-terminal region of the molecule. This is consistent with the findings of Pei et al. (33), who found that two proteins of different M_r from the same strain of *C. fetus* shared their N-terminal sequence. The mechanism by which a single strain can produce a range of S-layer proteins of different subunit M_r is unclear at this time but is likely to be novel,

especially if *C. fetus* is comparable to other reported S-layer-producing organisms in which a single chromosomal gene codes for the S-layer protein (3, 16, 25, 35, 48, 51).

Protease digestion of the purified VC119 protein under nondenaturing conditions provided convincing evidence for the presence of two domains in the *C. fetus* S-layer protein, a large trypsin-, chymotrypsin-, and endoproteinase Glu-C-resistant core with an M_r of approximately 110,000 and a smaller enzyme-sensitive domain with an M_r of approximately 20,000. Interestingly, the trypsin and chymotrypsin sites were within two residues of one another. Amino acid composition analysis of the 110,000-M_r chymotrypsin-resistant peptide confirmed the presence of lysine (trypsin cleavage site) and the aromatic amino acids phenylalanine and tyrosine (chymotrypsin cleavage sites), as well as aspartate and glutamate residues (endoproteinase Glu-C cleavage sites) (data not shown). Hence, the inability of these three enzymes to further cleave the 110,000-M_r fragment indicates that their cleavage target residues are not surface exposed and must be buried by virtue of conformation. The presence of an enzyme-resistant major structural domain may in fact be a feature common to S-layer proteins, as the S-layer proteins purified from *Aeromonas* species and *Lampopedia hyalina* also display a protease-resistant domain under nondenaturing conditions (1; T. J. Trust and W. W. Kay, unpublished observations).

The assembled *C. fetus* S layer was also thermostable, retaining immunoreactivity despite exposure of the cells to a temperature of 100°C for 10 min. In this characteristic the *C. fetus* S layer differs from the S layers of pathogenic *Aeromonas* species (13, 36), but while this thermophilic stability is perhaps unusual in the case of a mesophilic species such as *C. fetus*, unusual thermophilic stability is not uncommon among S layers. For example, the S layers of organisms such as *Sulfolobus acidocaldarius*, *Thermotoga maritima*, and *Methanothermus sociabilis* are all resistant to high temperature (12, 21, 24). However, these bacteria are capable of growth at thermophilic temperatures, in contrast to *C. fetus*, which grows at 37°C.

In summary, the ability of individual strains of a variety of pathogens to produce surface components with different antigenicities is a well-recognized virulence property (9). Indeed, antigenic variation of flagella may be an important virulence property of the enteropathogenic campylobacters (20, 28). This study has revealed another example of antigenic variability within the genus and the first example of the involvement of a true S layer in the antigenic variability of a single strain. Since the S layer covers the entire cell surface and is the immunodominant antigen of the cell, the ability to alter this gene product appears to be both a novel and an ideal defensive strategy for *C. fetus*.

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